Mitochondrial DNA and ITS1 Differentiation in Geographical Populations of Northern Corn Rootworm, *Diabrotica barberi* (Coleoptera: Chrysomelidae): Identification of Distinct Genetic Populations

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ABSTRACT Genetic variation of mitochondrial DNA (mtDNA) and the nuclear ribosomal spacer, ITS1, in local and dispersed geographical populations of northern corn rootworm, *Diabrotica barberi* Smith & Lawrence was examined. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used for mtDNA and DNA sequencing plus PCR-RFLP analysis was used for ITS1. Insects were collected in 10 states ranging from Pennsylvania to the Great Plains. Sequencing of the ITS1 amplicon revealed three potential polymorphic sites, one of which altered a restriction site for the restriction enzyme *Bcl*I. PCR-RFLP analysis with *Bcl*I detected three genotypes. Many beetles had heterogeneity at the nucleotide site recognized by *Bcl*I, which was supported by DNA sequence data. There appears to be a phylogeographic pattern of ITS1 genetic diversity. Eastern populations were homogeneous for one genotype, populations from central and northern locations had two genotypes, and western populations were composed of all three genotypes. The mtDNA had 58 haplotypes that displayed a strong east-west geographical partition. The region of overlap occurred in a few counties of east-central Illinois. Populations to the east had less variability than those to the west. A network of restriction site changes and trees based on genetic distance measurements of the mtDNA produced two distinct clades. One clade contained all the eastern haplotypes along with a group of haplotypes from the northern Great Plains. The other clade included the remaining western haplotypes. Possible reasons for this population structure including expansion from different glacial relicts, historic host plant differences, and endosymbiont driven reproductive incompatibilities are discussed.

KEY WORDS ITS1, mitochondrial DNA, polymerase chain reaction-restriction fragment-length polymorphism, population genetics, phylogeography

THE CORN ROOTWORMS, *Diabrotica* spp., are part of the large group of chrysomelid beetles, many of which attack agricultural crops. In the corn-growing regions of the Midwest and Great Plains, both the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, and the northern corn rootworm, *Diabrotica barberi* Smith & Lawrence are major pests. Soil insecticides applied for their control represent one of the major uses of insecticide in the United States. Annually, 20–25 million acres of corn are treated with soil insecticides to protect the crop from feeding damage by corn rootworm larvae. Costs associated with insecticides applied to control larval damage to corn roots and adult damage to corn silks, along with crop losses can approach $1 billion annually (Metcalf 1986).

Despite the possible benefits that molecular genetic analysis of northern corn rootworm may provide toward diagnostics, dispersal, insecticide resistance, and the implementation of area-wide control programs, little research in this area has been conducted. Previous population genetic analysis of northern corn rootworm did not reveal much genetic differentiation. An assessment of 33 northern corn rootworm populations using allozymes did not reveal any significant genetic variation within and among geographically isolated populations (McDonald et al. 1985). A similar study conducted by Krafsur et al. (1993) analyzing 21 polymorphic allozyme loci from 14 populations showed high levels of gene flow among *D. barberi* populations. Also, a study involving univoltine and semivoltine northern corn rootworm populations revealed gene flow to be virtually unrestricted between the diapause phenotypes based on allozyme data (Krafsur 1995).

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We have begun to examine genetic variation of mitochondrial DNA (mtDNA) and nuclear ribosomal DNA in local and dispersed geographical populations of northern corn rootworms. The goals include to survey population diversity and to determine whether molecular marker systems can be used for identifying strains and emerging phenotypes that might affect area wide control programs. We used polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) of large segments of the mitochondrial genome and we also examined the first Internal Transcribed Spacer (ITS1) region located between the repeating array of nuclear 18S and 5.8S ribosomal genes. We chose PCR-RFLP-based DNA sequence information for this study because the technique is relatively simple and inexpensive, very reliable and repeatable, and provides discreet character states that can be used for phylogenetic analyses and diagnostics (Taylor et al. 1999, Roehrdanz 2001).

Materials and Methods

Rootworms. Northern corn rootworm adults were obtained from the field locations listed in Table 1. State abbreviations used throughout are: North Dakota (ND), South Dakota (SD), Nebraska (NE), Kansas (KS), Iowa (IA), Wisconsin (WI), Illinois (IL), Indiana (IN), Ohio (OH), and Pennsylvania (PA). Samples were collected between 1997 and 2000. Except for the Nebraska collection, adult rootworms were shipped to the USDA–ARS Biosciences Research Laboratory in Fargo, ND. Some were transported live and frozen at −80°C upon arrival. Others were frozen before shipment and transported on dry ice. Frozen voucher specimens are being maintained at the USDA–ARS Biosciences Research Laboratory, Fargo, ND.

DNA Extraction and Amplification. DNA was extracted from individual insects using either the high-salt procedure of Cheung et al. (1993) or the Puregene DNA isolation kit D-5000A (Gentra, Minneapolis, MN) (Nebraska collection). Total genomic DNA served as the template for the long PCR of mtDNA. Long PCR reactions were done as described by Roehrdanz (1995). Mitochondrial primers included: C1 (C1-J-2195), C2 (C2-N-3662), N4 (N4-J-8944), 12S (SR-N-14588), CB2H (CB-N-10920) (Simon et al. 1994, Roehrdanz and Degrugillier 1998) and NCR-410 (C2-J-3250, 5′-TTTTTGCACCGAAATCTATTA-3′). The primary amplicons were 12S-N4 (≈5.5 kb) and CB2H-C2R (≈7.2 kb), which partially overlap. In some instances, the CB2H-C2R amplicon was weak and the CB2H-C1 or CB2H-NCR–410 primer pairs were used.

The ITS1 region was amplified using rDNA2 primer (5′-TTGATTACGTCCCTGCCCTTT-3′) (Vrain et al. 1992) and rDNA 1.58s (5′-GCCACCTAGTGAGC-CGAGCA-3′) (Cherry et al. 1997). These primers amplify a 3′ portion of the 18S gene, the entire ITS1 region, and a 5′ section of the 5.8S gene. PCR reactions were conducted using 1 μl of the extracted DNA, as described by Szalanski et al. (1997) with a profile consisting of 35 cycles of 94°C for 45 s, 54°C for 45 s, and 72°C for 60 s. Amplified DNA from individual beetles was purified, then concentrated using Microcon-PCR Filter Units (Millipore, Bedford, MA). Samples were sent to Davis Sequencing (Davis, CA) for direct sequencing in both directions using an ABI Prism 377 DNA sequencer. Consensus sequences were derived from the sequences in both directions using GCG Wisconsin Package software (Accelrys, San Diego, CA). GenBank accession numbers for the beetles subjected to DNA sequencing in this study are AY136647–AY136664. The DNA sequence of an individual beetle collected from Brookings, SD, GenBank AF155574, from a study by Szalanski et al. (1999, 2000), was also used.

Restriction Fragment-Length Polymorphism Analysis. Restriction sites were predicted from the ITS1 sequence data using Web Cutter 2.0 (Heiman 1997). Amplified ITS1 DNA was digested using the enzyme *Bcl*I (New England Biolabs, Beverly, MA). Restriction enzymes *Dpn*II, *Hin*fI, *Xba*I, and *Swa*I were used for the mtDNA amplicons. They have recognition sequences of 4 bp, 5 bp, 6 bp, and 8 bp, respectively. They were chosen because they demonstrated polymorphic fragment patterns and produced a number of fragments that could be conveniently scored. PCR ampli-

Table 1. Northern corn rootworm collection sites, mtDNA haplotypes and ITS genotypes

ID	State[a]	County	Nearest town	mtDNA			ITS1			
				N	(%) Haplotype (*N*)[b]	POP[c]	*N*[d]	A	B	A/B
FAR	ND	Cass	Fargo	9	**30** (3), **47** (1), **48** (1), **49** (1), **50** (1), **51** (1), **52** (1)	W	6	0	3	3
AUR	SD	Brookings	Aurora	10	**3** (1), **4** (1), **25** (1), **30** (3), **31** (1), **34** (2), **36** (1)	W	6	1	1	4
BRU	SD	Brookings	Bruce	13	**5** (1), **11** (1), **15** (1), **21** (1), **22** (1), **30** (6), **34** (1), **37** (1)	W	6	1	1	4
VOL	SD	Brookings	Volga	14	**1** (2), **2** (2), **6** (1), **7** (1), **19** (1), **23** (1), **29** (1), **30** (4), **33** (1)	W	5	2	3	0
465	SD	Brookings	Brookings	10	**1** (3), **2** (1), **30** (3), **32** (1), **33** (1), **35** (1)	W	6	2	3	1
BRK	SD	Brookings	Brookings	9	**1** (1), **6** (3), **9** (2), **30** (3)	W	0			
SCA	KS	Republic	Scandia	17	**1** (1), **2** (3), **6** (7), **19** (2), **24** (1), **26** (1), **28** (1), **29** (1)	W	13	4	3	6
CHP	KS	Dickinson	Chapman	7	**6** (1), **23** (6)	W	3	1	2	0

(Continued)

Table 1. Continued

ID	State[a]	County	Nearest town	mtDNA			ITS1			
				N	(%) Haplotype (*N*)[b]	POP[c]	*N*[d]	A	B	A/B
NEB	NE	Howard	Dannebrog	0		W	90	19	35	36
AME	IA	Story	Ames	22	**1** (11), **2** (7), **6** (2), **9** (1), **20** (1)	W	13	1	10	2
PSN	IA	Clinton	Preston	20	**1** (1), **2** (5), **8** (1), **13** (5), **14** (7), **17** (1)	W	5	0	1	4
COL	WI	Columbia	Arlington	8	**2** (2), **8** (4), **14** (1), **21** (1)	W	5	0	4	1
ROK	WI	Rock	Janesville	10	**8** (4), **14** (2), **21** (2), **29** (1), **44** (1)	9WIE	0			
WAR	IL	Warren	Monmouth	9	**1** (1), **2** (2), **8** (2), **29** (4)	W	6	0	6	0
KNX	IL	Knox		4	**1** (1), **8** (2), **44** (1)	3WIE	0			
AUG	IL	Knox	St Augustine	12	**2** (6), **8** (1), **14** (2), **29** (2), **44** (1)	11WIE	0			
STK	IL	Stark	Wyoming	8	**2** (7), **44** (1)	7WIE	6	0	5	1
HEN	IL	Henry	Anawan	1	**53** (1)	W	1	0	0	1
JOD	IL	JoDavies	Hanover	6	**1** (1), **2** (3), **14** (2)	W	5	0	3	2
MCH	IL	McHenry	Hebron	1	**2** (1)	W	1	0	1	0
SUG	IL	Kane	Sugar Grove	6	**2** (3), **55** (3)	W	0			
KNE	IL	Kane	Pinegree Grove	2	**2** (1), **29** (1)	W	2	0	2	0
KEN	IL	Kendall	Plattville	3	**25** (1), **29** (2)	W	0			
YRK	IL	Kendall	Yorkville	4	**2** (3), **29** (1)	W	0			
DOV	IL	Bureau	Dover	12	**2** (12)	W	5	0	4	1
PER	IL	Peoria	Laura	1	**14** (1)	W	1	0	1	0
LAS	IL	LaSalle	Cedar Point	8	**2** (1), **10** (4), **16** (1), **18** (2)	W	7	0	6	1
PAN	IL	Woodford	Panola	9	**2** (7), **56** (2)	W	5	0	5	0
MNK	IL	Woodford	Minonk	9	**2** (9)	W	0			
VAR	IL	Marshall	Varna	11	**2** (8), **15** (2), **29** (1)	W	5	0	5	0
GRN	IL	Grundy	Mazon	6	**2** (5), **29** (1)	W	0			
GAR	IL	Grundy	Gardner	11	**2** (11)	W	4	0	4	0
LIV[e]	IL	Livingston	Wing	1	**2** (1)	W	0			
WNG[e]	IL	Livingston	Wing	9	**38** (7), **44** (2)	E	0			
GRI	IL	Livingston	Gridley	10	**2** (10)	W	5	0	3	2
FLA	IL	Livingston	Flanagan	25	**2** (20), **17** (4), **29** (1)	W	0			
SAU	IL	Livingston	Saunemin	19	**2** (10), **17** (2), **29** (2), **38** (1), **41** (1), **56** (3)	17W1E1B	0			
CLK	IL	McLean	Clarksville	26	**2** (22), **29** (2), **57** (2)	W	0			
MCL[f]	IL	McLean	Cooksville	9	**2** (2), **17** (1), **41** (1), **43** (1), **44** (4)	3W4E2B	7	0	7	0
COK[f]	IL	McLean	Cooksville	9	**2** (8), **29** (1)	W	0			
LOG[g]	IL	Logan	Lincoln	4	**2** (3), **12** (1)	W	0			
LIN[g]	IL	Logan	Lincoln	11	**2** (4), **15** (7)	W	5	0	4	1
DEW	IL	DeWitt	Kenney	8	**2** (7), **29** (1)	W	6	0	2	4
MID	IL	DeWitt	Midland City	12	**2** (11), **29** (1)	W	5	0	5	0
CLI	IL	DeWitt	Clinton	2	**15** (1), **38** (1)	1WIE	0			
WEL	IL	DeWitt	Weldon	1	**58** (1)	E	0			
HAL	IL	DeWitt	Hallsville	2	**15** (2)	W	0			
FOR	IL	Ford	Sibley	9	**30** (2), **38** (4), **40** (1), **44** (2)	2W6EIB	8	0	8	0
PDU	IL	Ford	Perdueville	10	**38** (8), **44** (2)	E	0			
CHM	IL	Champaign	Urbana	15	**38** (11), **44** (4)	E	9	0	9	0
PAT	IL	Piatt	Deland	8	**39** (1), **44** (6), **45** (1)	E	0			
BEM	IL	Piatt	Bement	11	**38** (4), **44** (7)	E	5	0	5	0
MON	IL	Piatt	Monticello	5	**38** (1), **44** (4)	E	0			
APK	IL	Piatt	Allerton Park	2	**38** (1), **44** (1)	E	0			
CEG	IL	Piatt	Cerro Gordo	10	**38** (3), **44** (6), **60** (1)	E	0			
PNA	IL	Piatt	Pierson/Atwood	11	**38** (8), **44** (3)	E	5	0	5	0
MAC	IL	Macon	Warrensburg	8	**41** (2), **44** (6)	6E2B	6	0	6	0
HAR	IL	Macon	Harristown	12	**2** (1), **15** (2), **44** (8), **59** (1)	3W8E1B	0			
MLT	IL	Moultrie	Lovington	8	**38** (7), **44** (1)	E	8	0	8	0
ARC	IL	Douglas	Arcola	11	**38** (11)	E	5	0	5	0
IRQ	IL	Iroquois	Sheldon	24	**38** (22), **42** (1), **44** (1)	E	12	0	12	0
ARM	IL	Vermillion	Armstrong	10	**38** (6), **44** (3), **61** (1)	E	5	0	5	0
NWT	IN	Newton		5	**38** (4), **44** (1)	E	0			
WLS	IN	Wells		4	**44** (4)	E	2	0	2	0
JAS	IN	Jaspar		2	**38** (2)	E	0			
TIP	IN	Tippecanoe	LaFayette	3	**38** (3)	E	2	0	2	0
MIA	OH	Miami	Troy	7	**38** (5), **44** (2)	E	5	0	5	0
RSP	PA	Centre	Rock Springs	12	**44** (11), **46** (1)	E	5	0	5	0
Totals				603			312	31	237	74

[a] State abbreviations as in Materials and Methods.

[b] Haplotype numbers in bold are as described in Table 3. Numbers in parentheses are the number of individuals with the haplotype at that location.

[c] W, western; E, eastern; B, restricted to boundary region, based on geographical segregation of haplotypes.

[d] Except for the NE collection, all insects assayed for ITS1 were also examined for mtDNA haplotype.

[e] LIV and WNG are collections made from the same site in different years.

[f] MCL and COK are collections made from the same site in different years.

[g] LIN and LOG are collections made from the same site in different years.

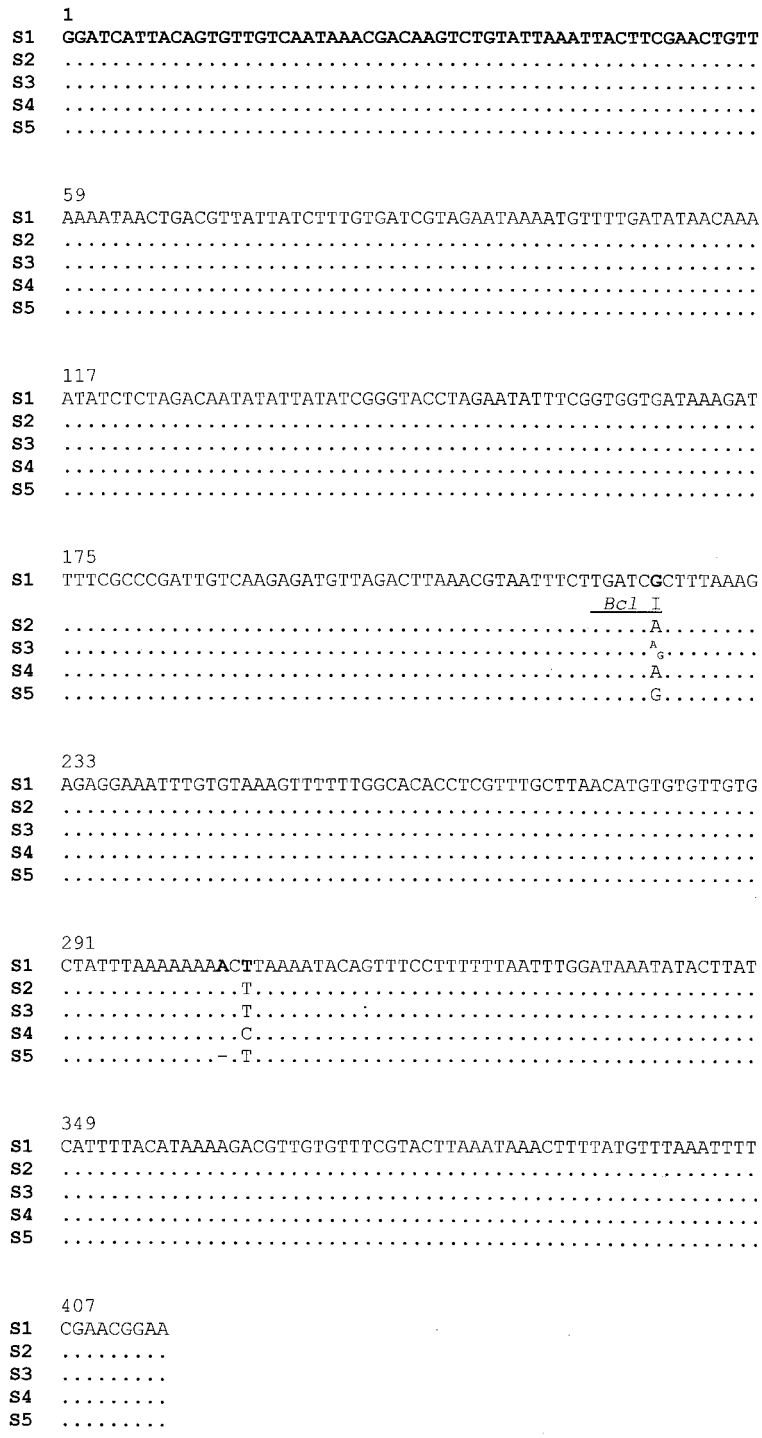

Fig. 1. DNA sequence alignment of ITS1 from northern corn rootworm beetles. The position of the *Bcl*I restriction site is indicated. Ribosomal gene regions from either end of the PCR product have been deleted. Dots, identical nucleotides; dash, indicates a missing nucleotide. The number of individuals with each sequence is: S1 (7); S2 (2); S3 (1); S4 (1); and S5 (8). Position 224 of S3 had peaks for both A and G and produced both *Bcl*I restriction patterns as shown in Fig. 2.

cons and digest products were separated and visualized via gel electrophoresis with ethidium bromide staining using agarose (1–1.8%) or metaphor (2.5–3%) for the mtDNA and polyacrylamide (10%) for ITS1. The gels were photographed and the picture here is a negative image of the ethidium fluorescence. Different restriction patterns were assigned a letter as they were observed. Each individual insect was assigned a composite haplotype based on the RFLP patterns. The haplotype network was constructed by using the restriction fragment data to estimate the minimal number of restriction site changes necessary to convert between the more common haplotypes. Genetic distances for each pairwise combination of composite haplotypes were calculated using the program Restsite (Miller 1991) and the PHYLIP Phylogeny Inference Package (Felsenstein 1993). Restsite performs an internal bootstrap and permits direct analysis of restriction fragment data generated by enzymes with differing recognition site lengths. The output is a set of distance matrices, one for each category and restriction enzyme and one for the combined data, that were used to create unweighted pair-group method with arithmetic average trees. The Phylip package (Seqboot, Restdist, Neighbor, and Consense) preserves bootstrap percentages for the tree branches, however, it can only deal with one restriction enzyme category at a time. Because there are four categories of restriction enzymes, each individual tree uses only a fraction of the data. In an effort to obtain a tree with bootstrap numbers, data from the different enzymes was combined but entered into the program as if it all had the same restriction site length. We ran the programs first assuming a 4-bp restriction site, then assuming a 5-bp restriction site, followed by 6 bp and 8 bp. The 25 haplotypes recovered more than once were used for this process. The trees were drawn with PHYLIP's Drawgram program.

Results

DNA sequencing of the ITS1 PCR-amplified product from 19 individual beetles representative of 14 populations revealed a size range from 645 to 646 bp among the northern corn rootworm DNA sequences. Trimming off the rRNA sequence leaves an ITS1 of 414–415 bp. Of the 19 northern corn rootworm individuals sequenced, three nucleotide sites (positions 224, 304, and 306) were polymorphic, and were found exclusively in the ITS1 region (Fig. 1). The polymorphism at site 306 was found only once in the sequence that was part of an earlier study (Szalanski et al. 1999). The polymorphism at site 304 varied the length of a run of adenines from 7 to 8. Eight of the sequences had the shorter adenine string. Site 224 was an A-G transition.

Restriction maps of the ITS1amplicons generated using Webcutter 2.0 (Heiman 1997) revealed that the restriction site for *Bcl*I recognized the polymorphism at nucleotide site 224. Beetles with two restriction sites and adenine at site 224 (275-, 243-, and 128-bp fragments) were assigned genotype "ITS-A." Beetles with

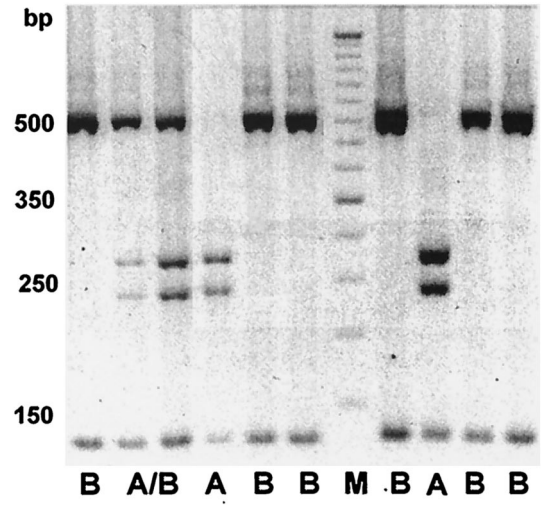

Fig. 2. PCR-RFLP digest of ITS1 using the restriction enzyme *Bcl*I on northern corn rootworm beetles. Genotype patterns are designated. M is a 50-bp molecular weight ladder. Image is a negative of ethidium bromide stained gel.

one *Bcl*I site and guanine at site 224 (517- and 128-bp fragments) were designated as genotype "ITS-B." (Figs. 1 and 2). Some restriction digests contained both fragment patterns. The occurrence of both types within an individual, designated as "ITS-A/B," was attributed to heterogeneity within individuals and analysis of DNA sequence electropherograms supports this conclusion. At site 224, analysis of the electopherogram of an insect from the SCA collection in Kansas clearly reveals sequence peaks for both adenine and guanine. That same individual also had the combined restriction fragment patterns. The RFLP patterns were used to screen additional 293 insects (Table 1).

Genotype ITS-B was the only genotype found in eastern populations (Indiana, Ohio, and Pennsylvania) (Fig. 3). Among the 24 counties sampled in Illinois, genotype ITS-B is prevalent in the eastern counties, whereas both genotypes ITS-B and ITS-A/B occur in western counties of Illinois (Table 1). In North Dakota and Wisconsin, only genotypes ITS-B and ITS-A/B occur in the two sampled locations. Genotype ITS-A was only found in South Dakota, Nebraska, Iowa, and Kansas and was less common than genotypes ITS-B and ITS-A/B, which also occurred in these four states (Fig. 3).

The two mtDNA segments comprised ≈70–75% of the mtDNA. The four restriction endonucleases yielded 51 restriction fragment patterns for the 12S-N4 and CB2H-C2R amplicons (Table 2). Approximately 50 restriction fragments per individual (116 total fragments) have contributed to the RFLP comparisons and 58 mtDNA haplotypes have been identified from 603 individuals (Table 3). Polymorphism has been observed both within populations and between collection sites. The haplotypes and numbers from each of the 68 collections (66 locations) are included in

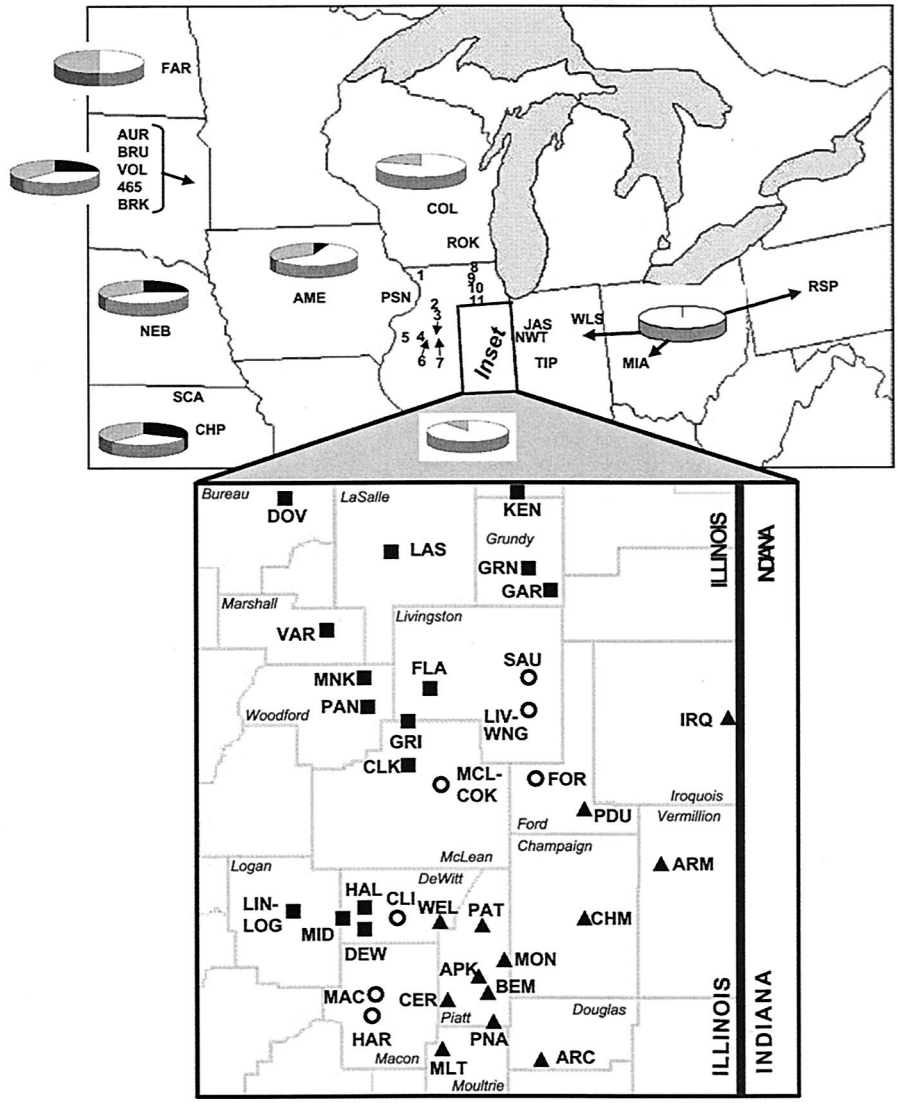

Fig. 3. Map of northern corn rootworm collections. Collections abbreviations correspond to Table 1 except in part of Illinois where number have been used because of space constraints. For that region: 1 = JOD; 2 = HEN; 3 = STK; 4 = KNX; 5 = WAR; 6 = AUG; 7 = PER; 8 = MCH; 9 = KNE; 10 = SUG; 11 = YRK. The numbers are at the approximate location of the collection site. In cases in which crowding of the numbers was an issue, arrows point to locations. Inset is enlargement of mtDNA haplotype boundary region. Solid squares are geographically western haplotypes, solid triangles are geographically eastern haplotypes, and open circles are locations that had both eastern and western haplotypes. Illinois county names are in italics. Pie charts are the frequency of ITS1 genotypes summed for each state. Black = genotype ITS-A, gray = genotype ITS-A/B, white = genotype ITS-B.

Table 1. More than one half of the haplotypes (33/58) have been recovered only once. The three most frequent haplotypes (2, 38, 44) account for 64% of the individuals and the seven haplotypes that have been observed 10 or more times comprise 85% of the individuals.

The haplotypes divide into two geographical clusters with a demarcation zone in east central Illinois (Fig. 3). The eastern population zone is east of a line extending from the southern end of Lake Michigan southwest to approximately Decatur, IL, and includes insects from Illinois, Indiana, Ohio, and Pennsylvania. West of the line, a different set of haplotypes takes over. Seven locations along the boundary (open circles on Fig. 3) can be described as having a mixture of eastern and western haplotypes. Four low-frequency haplotypes were restricted to that region and cannot be characterized as eastern or western. Only nine haplotypes are found in the eastern zone with haplotypes 38 and 44 being 96% of the individuals including

Table 2. Mitochondrial restriction fragment patterns

PCR product	Enzyme	Pattern	Fragment sizes (bp)
12S-N4	*Dpn*II	A	1250, 1200, 700, 550, 460, 430, 300, 240, 220, 160
		B	1250, 1200, 700, 550, 460, 300, 280, 240, 220, 160, 135
		C	1250, 1200, 700, 550, 460, 300, 240, 175, 160, 100
		D	1450, 1200, 700, 550, 460, 430, 300, 240, 220
		E	1250, 1200, 700, 550, 460, 300, 240, 220, 175, 160
		F	1250, 1200, 700, 680, 550, 460, 240, 220, 160
		K	1250, 1050, 700, 560, 460, 430, 300, 240, 220, 160, 150, 100
	*Hin*fI	A	1900, 1400, 700, 600, 440, 430
		B	1900, 1100, 700, 600, 440, 430, 325
		C	1400, 1100, 1000, 700, 600, 440, 430
		D	1400, 1200, 850, 700, 600, 440, 430
		E	2300, 1400, 700, 600, 440
		G	1700, 1400, 1000, 700, 440, 430
	*Swa*I	A	~3000, 700, 640, 355, 320, 165, 120
		B	~3000, 640, 460, 355, 320, 280, 165, 120
		C	~2800, 640, 460, 370, 355, 320, 280, 165, 120
		D	~3000, 720, 700, 355, 320, 165,
		E	~3000, 750, 700, 355, 320, 120
		F	~3000, 750, 700, 355, 320, 240, 120
		G	~3000, 700, 640, 355, 210, 165, 120
	*Xba*I	A	>4500, 450
		B	>4500, 450, 400
		C	2400, 1900, 450, 400
		D	4100, 450, 440, 400
CB2H-C2R	*Dpn*II	A	3000, 1450, 1200, 360, 290, 230
		B	3000, 1200, 900, 440, 360, 290, 230
		C	2000, 1450, 1200, 950, 700, 360, 290, 230
		D	2900, 1450, 1200, 700, 360, 290, 230
		E	3000, 1200, 750, 440, 360, 290, 230, 165
		F	2400, 1200, 1000, 900, 440, 360, 290, 230
		G	3500, 1000, 900, 440, 360, 290, 230
		H	3000, 1250, 1200, 440, 360, 290, 230
	*Hin*fI	A	4200, 1450, 720, 500, 450
		B	3100, 1200, 1150, 1140, 450, 300
		C	4200, 1450, 800, 450, 400
		D	4200, 1450, 1200, 450
		E	3100, 1450, 1200, 1100, 450
		G	3100, 1450, 1200, 1150, 450
		H	3100, 1200, 1150, 720, 550, 450
		J	2300, 1800, 1450, 1200, 450, 210
		N	3100, 2300, 1200, 450, 300
	*Swa*I	A	1750, 1500, 1450, 1400, 680, 640, 475, 355, 200, 170
		B	1750, 1500, 1450, 1400, 780, 680, 475, 355, 200
		C	3000, 1750, 1400, 680, 640, 475, 355, 200, 170
		E	1750, 1500, 1450, 1250, 680, 355, 200, 170
		G	1800, 1750, 1500, 1450, 680, 640, 355, 200, 170
	*Xba*I	A	4000, 1800, 1100
		B	2400, 1800, 1500, 1100
		C	5800, 1100
		D	3000, 2400, 1500
		F	2600, 800, 1200, 1100, 440

those in the boundary area. The other seven eastern haplotypes were singletons. Four individuals with eastern haplotype 44 spill over into the western regions, three in western Illinois and one in Wisconsin. The western zone is more diverse, containing 46 haplotypes. Here haplotype 2 is predominant at 49%. None of the western haplotypes were found east of the boundary zone. The boundary is sharp with the transition from the eastern population to the western population occurring within the space of ≈30 miles. Two of the sites along the boundary, LIV-WNG and MCL-COK, had insects from two years, 1998 and 1999. LIV had a lone western haplotype in 1998, whereas the WNG sample from 1999 was all eastern. At the McLean County site, the MCL collection from 1998 had a mixture of western, eastern, and boundary haplotypes, but the 1999 COK collection was all western (Table 1). The geographical characterization of the haplotypes from all of the collection sites is in column seven of Table 1. None of the western haplotypes were found east of the boundary zone, however four of 83 individuals from eastern haplotype 44 were obtained from west of the boundary.

A network was constructed connecting the most numerous haplotypes, those found >5 times, in a fashion to minimize the number of restriction site changes among the haplotypes (Fig. 4). The smallest circle corresponds to seven individuals whereas the largest represents 193 individuals. Two groups, centered around haplotype 2 and haplotype 44, are apparent. At

Table 3. Restriction fragment patterns associated with each mtDNA haplotype

Haplotype #[a]	Amplicon		N	Haplotype #[a]	Amplicon		N
	12S-N4[b]	CB2H-C2R[b]			12S-N4[b]	CB2H-C2R[b]	
1	AAAA	AAAA	22	31	BCBB	BBAA	1
2	AAAA	ADAA	193	32	BCBB	AAAB	1
3	AAAA	DDAC	1	33	BCBB	BHAB	2
4	AAAA	ADAD	1	34	BCBB	ABAB	3
5	AAAA	AGCA	2	35	BCBA	BBAA	1
6	AAAA	DCAA	14	36	FCBB	DBAB	1
8	AAAA	ACAA	14	37	BADA	BCEA	1
9	AAAA	CCAA	3	47	CEAA	AACA	1
10	AAAA	BDAA	4	48	AEAA	AGCA	1
11	AAAA	BCAA	1	49	AEGA	ADAA	1
12	AAAA	ADGA	1	50	AEAA	DCAA	1
13	AAAA	DJAC	5	51	BCBC	BBAB	1
14	AAAA	FJAC	15	52	BDBB	BBAB	1
15	ABAA	ADAA	15	53	AAAA	ADAC	1
16	ABAA	BDAA	1	55	BBAA	ADAA	3
17	AABA	ADAA	7	56	BAAA	ADAA	7
18	AABA	BDAA	2	38	ACCB	BBAB	108
19	AAEA	AABA	3	44	ACBB	BBAB	83
20	AEAA	AAAA	1	39	ACAB	BBAB	1
21	BAAA	BDAC	4	40	AACB	BBAB	1
22	BAAA	ACAA	1	41	ACAA	ADAA	4
23	CAAA	BEAC	7	42	ACCA	BBAA	1
24	CAAA	EEAC	1	43	ACBA	ADAA	1
25	DAAA	ADAA	2	45	ACBB	HBAB	1
26	EAAA	DCAA	1	46	ACBB	ABAB	1
27	BAAA	AEAA	1	58	ACBB	BBAD	1
28	ADAA	ACAA	1	59	ACBB	BBGB	1
29	DBAA	ADAA	22	60	ACBD	BBAF	1
30	BCBB	BBAB	24	61	KGCB	GNAB	1

[a] Some haplotype numbers are missing because those haplotypes were later merged with preexisting haplotypes.
[b] Patterns are for the restriction enzymes, *Dpn*II, *Hin*fI, *Swa*I, and *Xba*I in that order.

least seven restriction site changes separate those two haplotypes. They are comprised of one *Swa*I site, one *Dpn*II site, two *Xba*I sites, and three *Hin*fI sites. The *Swa*I site gain relative to haplotype 2 results in the intermediate haplotype 17. Two apparent homoplasies were also noted.

Genetic distances between haplotypes were determined first with the 25 haplotypes that occurred more than once and then incorporating all of the haplotypes. This information was used to construct the trees. Two distinct clades, A and B, divide the haplotypes. The haplotypes fall into the same groups as they did with the network of restriction site changes. The bootstrap numbers for the two main branches are both >95% (Fig. 5). Arrangements within the two branches have low bootstrap support. When all of the haplotypes are considered, branch A is composed of 35 western haplotypes and 2 haplotypes from the boundary region. All of the eastern haplotypes are on branch B. Clade B also includes two boundary haplotypes and nine western haplotypes (Fig. 6). These nine western haplotypes all originated from the northern Great Plains in North and South Dakota. Thus, the closest relatives of the eastern haplotypes are not from the region adjacent to the border, but are from the geographically distant Dakotas. A map shows the distribution of individuals from the two clades (Fig. 7).

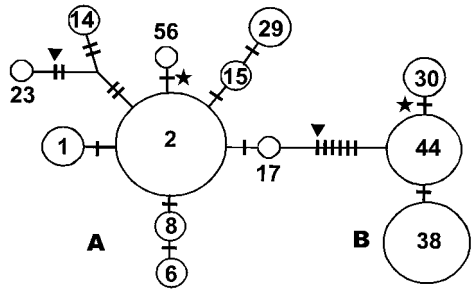

Fig. 4. Haplotype network connecting the northern corn rootworm haplotypes that were recovered from >5 individuals. Haplotype numbers are as given in Table 3. The area of the circles is proportional to the number of individuals with that haplotype (see Table 3). Bars on the connecting lines indicate the minimal number of restriction site changes observed. Triangle, *Hin*fI homoplasy; star, *Dpn*II homoplasy. A and B = two distinct groups.

Discussion

Low genetic variation has been documented for both subspecies of another diabroticite, western corn rootworm and Mexican corn rootworm (*Diabrotica virgifera zeae* Krysan and Smith). No ITS1 or mtDNA markers were found that were diagnostic for the subspecies despite the fact that there are obvious color differences. What little polymorphism that was detected had no geographical component (Szalanski et al. 1999). This contrasts with the northern corn rootworm findings.

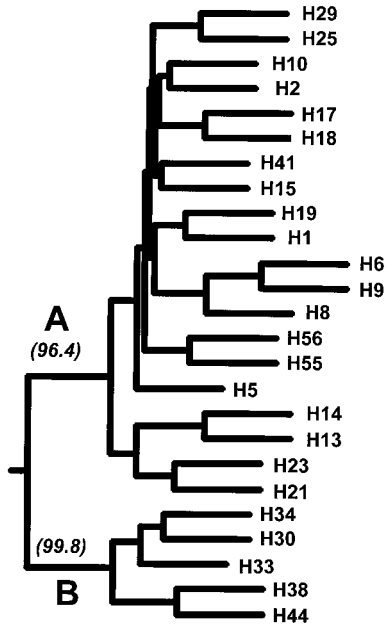

Fig. 5. Consensus tree of all haplotypes recovered more than once. Bootstrap values are indicated for the two clades, A and B. Haplotype numbers as in Table 3. Tree created with Phylip.

Previous investigations of variability in northern corn rootworm have demonstrated some polymorphism with a hint of geographical differentiation. Krysan et al. (1983) and Krysan and Smith (1987) have stated that northern corn rootworm from Massachusetts, New York, and Pennsylvania have noticeably darker legs and antennae with the color becoming paler in the western part of the range. However, the chromatic cline is rather patchy. Beetles from New Jersey were similar to those from the Midwest, insects from Atlantic Canada were as light as those from Kansas and Nebraska, and beetles from South Carolina and Georgia were dark. The color differences were more easily observed in live beetles than they were in dead specimens. This chromatic variability raised the possibility that there may be genetically distinct populations. McDonald et al. (1985) have shown evidence of "eastern" and "western" geographical races of northern corn rootworm based a combination of coloration and allozyme variation. They used the same color characters as Krysan et al. (1983) and found a similar distribution. The darkest insects were from Massachusetts, New York, New Jersey, and Pennsylvania. Beetles from southern Ontario adjacent to New York and ones from Virginia were lighter like those from Ohio and Illinois. They also reported polymorphism in seven enzyme systems, although it should be noted that except for hexokinase and isocitrate dehydrogenase the second most frequent allele was usually <3%. The percentage of population variability was higher for eastern subpopulations than western ones, except for Kansas and Nebraska, which were the highest of all. Genetic distance measurements also set off the Kansas and Nebraska samples as a separate group. However, the authors could not exclude the possibility that some of the Kansas and Nebraska collections included the closely related species, *D. longicornis* Say. The remaining samples from South Dakota to Massachusetts clustered in a group with Ohio at the center and each of the sites separated from one or members of the cluster by a genetic distance of <0.005. Overall, there was little evidence for discrete populations. A similar study conducted by Krafsur et al. (1993) analyzing 21 polymorphic loci from 14 populations, showed high levels of gene flow, i.e., no differentiation, among *D. barberi* populations in Iowa. Also, a study involving univoltine and semivoltine northern corn rootworm populations revealed gene flow to be virtually unrestricted between the diapause phenotypes based on allozyme data and therefore no evidence of differentiation based on this behavioral trait (Krafsur 1995).

Although the sequence determination uncovered three potential polymorphisms, only the polymorphism at site 224 has been used for population comparisons. The gain or loss of an extra A at position 304 and the substitution found only once at position 306 were not diagnostic and can be attributed to sequencing anomalies.

Unlike previous population genetic studies on northern corn rootworm, ITS1 DNA sequencing and PCR-RFLP analysis of ITS1 and mtDNA have revealed genetic variation within and genetic differentiation among northern corn rootworm populations. With ITS1 there appears to be a phylogeographic pattern of genotype frequencies, with eastern (eastern Illinois, Indiana, Ohio, Pennsylvania) populations exclusively type ITS-B and western populations with a mixture of type ITS-B and ITS-A/B genotypes (western Illinois, Wisconsin, North Dakota) or all three observed genotypes (South Dakota, Nebraska, Iowa, and Kansas). A gradual delineation occurs in Illinois with genotype ITS-B most prevalent in eastern counties and genotypes ITS-A/B and ITS-B occurring in western counties. The boundary between populations exclusively ITS-B and the appearance of ITS-A/B heterozygotes appears to be somewhere in east-central Illinois.

ITS1 heterogeneity within individuals has been observed in mosquitoes (Wesson et al. 1992) and nematodes (Cherry et al. 1997, Szalanski et al. 1997). As with our study, regional differentiation in intragenomic heterogeneity of the rDNA ITS region has been observed in *Anopheles nuneztovari* (Onyabe and Conn 1999). They observed regional differentiation in intragenomic heterogeneity in *A. nuneztovari* between both Venezuela and Colombian samples versus the Brazilian samples and concluded that it probably reflects either differences in population-level processes such as gene flow and genetic drift, or the fact that these localities may represent two or more cryptic species. The experiments reported here cannot distinguish between variations among copies derived from a single locus of the ribosomal repeating array of genes, the presence of a second variant ribosomal locus, or two ribosomal alleles constituting a riboso-

Fig. 6. Tree of all mtDNA haplotypes. Haplotype numbers as in Table 3. Number of individuals and type in parentheses. W = western, E = eastern, B = boundary found only at one of the open circle sites of Fig. 3 inset. Large A and B denote the two clades. Unweighted pair-group method with arithmetic average tree from Restsite.

mally heterozygous individual. Regardless of the underlying structural basis for the polymorphism, it is apparent that PCR-RFLP analysis with *Bcl*I is a useful marker for northern corn rootworm.

The mtDNA long PCR-RFLP results are even more striking. When the results from insects collected in 1998 and 1999 were assembled and it appeared that there was a geographic pattern to the haplotype distribution, the three most frequent haplotypes were falling into two groups that we categorized as east and west. It became clear that east-central Illinois was the key location separating these haplotypes. A plan was developed to make more extensive collections across what we thought was the boundary between divergent haplotypes. In 2000, six additional collection sites were sampled across Ford, Livingston, and McLean Counties (PDU, COK, WNG, SAU, FLA, CLK), and eight more sites from Piatt, Macon, and DeWitt Counties (BEM, MON, APK, CER, HAR, WEL, CLI, HAL). These proved crucial in defining both the location and extent of the haplotype boundary. The locations that contained both eastern and western haplotypes fall in a fairly narrow corridor from SAU south to HAR. This zone of overlap could be as much as 30 miles (50 km) wide in the FOR-MCL area and as little as 10 miles (15 km) or less around CLI.

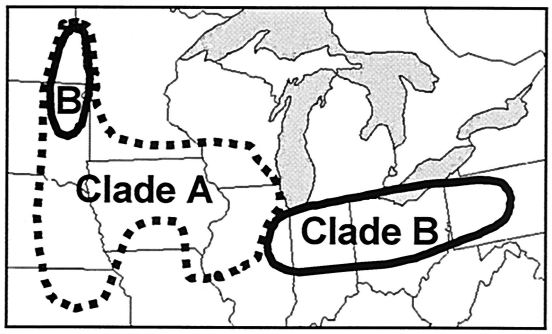

Fig. 7. Geographic distribution of the clade A (dotted line) and clade B (solid line).

The genetic distance determinations and the restriction site change network both support the separation of these two populations in Illinois. Branches A and B of the trees reflect a sequence divergence of ≈2.3%. That level of divergence is similar to that between screwworm (*Cochliomyia hominivorax*, Coquerel) populations on the mainland of Central America and the island of Jamaica (Roehrdanz 1989). In the case of the screwworm, a large body of water creates a physical barrier isolating the populations. For the northern corn rootworm, there is no comparable physical barrier. The two genetically divergent populations abut each other but there is almost no mixing. Just as there were no western haplotypes obtained from east of the border zone, no eastern haplotypes were found on branch A of the tree. Of the hundreds of insects examined, only four, all eastern haplotype 44, were collected from both sides of the boundary. Because they were identified from single insects at four different locations in Illinois and Wisconsin, it would require testing a large number of insects from those sites to determine if small viable populations exist at those sites or if the insects collected were merely accidental residents. All four locations were near major interstate highways, so the potential exists for hitchhiking as a means of dispersal.

Both haplotype frequencies and genetic distance support the conclusion that the eastern population has lower overall genetic variability than the western population. In addition to the fact that 96% of the 194 eastern insects examined were haplotypes 38 or 44, these two haplotypes diverge by a single restriction site or only 0.3%. The western group has not only five times as many identified haplotypes but greater divergence between some of the more frequent ones. Haplotype 2 differs from haplotype 14 by at least four sites or 0.6%. Furthermore there are the nine western haplotypes comprising 35 insects that are on branch B with the 2.3% average divergence from the other western haplotypes. This group is an unexpected enigma. They are genetically most closely related to the eastern haplotypes, yet 33 of 35 insects were obtained from North Dakota and South Dakota in the far northwest corner of the northern corn rootworm range (see Fig. 7). Two insects of haplotype 30 were obtained from the boundary zone in Illinois. Although haplotype 30 is the most numerous haplotype in North Dakota and South Dakota, ≈33%, it does not dominate the population the way haplotypes 38 and 44 do in the east. There were 21 other haplotypes from branch A also found in North Dakota and South Dakota marking those collection sites as containing the greatest diversity. One possibility for the cluster of branch B haplotypes in the Dakotas is that they are descendants of individuals that were artificially transferred from the east perhaps with human assistance. Another scenario is that clade B was once distributed across the entire range of northern corn rootworm but was eliminated from the middle of the range by lineages that obtained some selective advantage.

The genetic distance data represented on the tree (Fig. 6) lets us assign the four haplotypes that were found only in the boundary zone. Haplotypes 41 and 43 are positioned on branch A, therefore it seems safe to conclude that haplotypes 41 and 43 are also western. By contrast, haplotypes 40 and 59 are embedded in the cluster of eastern haplotypes on branch B. Haplotypes 40 and 59 can legitimately be labeled as eastern. The final tally of haplotypes is 11 eastern and 48 western.

Some of the more common restriction fragment patterns have proven to be diagnostic, or nearly so, for clade A versus B or for western versus eastern populations. For the 12S-N4 amplicon, *Xba*I patterns B, C, and D were found only in clade B western individuals; 368 of 370 *Xba*I pattern A were from clade A, and all but one were western; 353 of 354 *Swa*I pattern A were clade A and western; 315 of 316 *Hin*fI pattern A were clade A and western whereas 231 of 236 *Hin*fI pattern C were clade B. For the CB2H-C2R amplicon, all 230 *Xba*I pattern B were clade B; 366 of 369 combined *Xba*I patterns A and C were clade A, and only one of these was eastern; all 231 *Hin*fI pattern B were clade B; all 271 pattern D, all 37 pattern C, all 20 pattern J, all 9 pattern E, and 27 of 28 pattern A *Hin*fI were clade A and all 365 were western. This information could simplify future investigations into whether the boundary is stationary or moving. Using *Hin*fI digests of the CB2H-C2R amplicon alone, only 1 of 603 individuals would have been assigned to the wrong clade, and the exception was collected in South Dakota far from the boundary.

The overall level of polymorphism, and especially the 2.3% divergence between the two clades, indicates a species that has been at home in this part of the country for a long time. This is in marked contrast to the western corn rootworm that rapidly expanded its range in the 20th century and displays very little genetic diversity (Szalanski et al. 1999). The molecular genetic difference between the northern corn rootworm genotypes is greater than anything found between western and Mexican corn rootworm. The genetic divide in Illinois does not correspond to the previous colorimetric and isozyme analyses of Krysan et al. (1983) and McDonald et al. (1985). Those reports both described gradual east-west clines. There was no clear-cut boundary separating populations.

What boundary they found was diffuse and further to the east.

The origins of the northern corn rootworm divergence are purely speculative. Perhaps the populations became adapted to different habitats, e.g., mixed woodlands versus prairie. The genetic demarcation could be reflective of adaptation to host plants before a shift to corn. *Diabrotica barberi* is a member of the virgifera species group of Diabrotica. This group relies on members of the grass family Poaceae as a host plant for larval development. Northern corn rootworm is known to complete its life cycle on at least 14 Poaceae grass host species other than corn (Branson and Ortman 1967, 1971). Unfortunately, these were all experimental studies that define a potential to use those plants as hosts. There is no reliable data on what host plants are or were actually used in the wild except for corn. Members of the grass family are widespread. They were once the dominant species on the great prairies of the central United States but are not restricted to that biome. It is possible that in the evolutionary history of northern corn rootworms the eastern and western groups developed a preference for different species of grass. Such a host plant divergence could result in reduced gene flow between the two populations. A related and not mutually exclusive possibility is that the populations derive from different geographic sources after the last round of glaciation. The lower diversity in the eastern population could stem from a genetic bottleneck such as a smaller glacial refugium.

Acquisition of other behavioral traits could serve as well to separate the populations, or such traits could arise and spread in one population but not the other. The unusual trait that has been studied most extensively in northern corn rootworm is prolonged or multi-year diapause. Typically northern corn rootworm eggs diapause through a single winter, however, researchers have observed eggs that successfully undergo two to four diapause seasons and retain their viability (Krysan et al. 1986, Levine et al. 1992). This phenomenon has been most noticeable in the northwestern portion of the northern corn rootworm's range (South Dakota, northwest Iowa, southwest Minnesota) where it has disrupted 2-yr crop rotations as a method for controlling the insects. However, eggs collected in Illinois exhibit just as high a frequency of multiyear diapause, but they are currently not a major concern to corn growers because the western corn rootworm is the dominant species in that region. Prolonged diapause does not seem to be limited to one of the two clades because eggs from both sides of the current genetic divide in Illinois exhibited the behavior (Levine et al. 1992). The caveat is that the eggs in the diapause studies were collected in 1986 and we do not know whether the genetic boundary was in the same place that we observed it in 1998–2000. Further research involving additional samples and known voltinism is required for a definitive resolution of this issue.

The sharp boundary associated with the mtDNA is reminiscent of the western corn rootworm's contact zone with the Mexican corn rootworm. That boundary is reinforced by a *Wolbachia* bacterial endosymbiont, resident in western corn rootworm but absent from the Mexican corn rootworm, that confers a reproductive barrier to matings between *Wolbachia*-infected and *Wolbachia*-uninfected beetles (Giordano et al. 1997). *Wolbachia*-infected sperm fertilizing uninfected eggs typically results in few or no offspring (cytoplasmic incompatibility). *Wolbachia* can also alter the sex ratio of a species, usually by killing male embryos. In principle, *Wolbachia* could act as an agent to isolate two populations, infected and uninfected, or it could be the agent that limits introgression between two populations that became isolated for other reasons. Because both *Wolbachia* and mtDNA are maternally inherited, *Wolbachia* infection is often associated with reduced mtDNA variability. Could a *Wolbachia* infection be responsible for the clearly delineated east-west separation of northern corn rootworm? We have recently obtained evidence that some northern corn rootworms harbor *Wolbachia* and we are in the process of determining the nature and extent of those infections (unpublished data).

The molecular genetic differences we describe here have not been specifically correlated with any behavioral differences but they do raise a caution flag. Researchers in both the eastern and western regions need to be alert to the possibility that quantitative or qualitative phenotypic differences may exist between the two populations of northern corn rootworm. This is especially relevant given the impending release of genetically modified corn with the *Bacillus thuringiensis* var. *tenebrionis* gene, which encodes for an endotoxin specific for Coleoptera, and is targeted toward corn rootworms. The implications of this genetic demarcation could influence the spread of insecticide resistance genes if it developed in beetles from one clade. There is also the potential for the two populations to respond differently to other control measures.

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